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Telomere length is tightly regulated in *Saccharomyces*. This lengthening is dependent on *TLC1*, which encodes the RNA component of telomerase but independent of *RAD52*, which encodes a protein required for most recombination events in mitotic yeast cells. Most cells lacking yeast telomerase die but survivors arise by *RAD52* dependent events: the majority of these survivors have tandem duplications of the sub-telomeric Y' element. However, about 10% of the survivors have a different telomeric structure, very long tracts of $C_{1-3}A/TG_{1-3}$ DNA, with some telomeres being as much as 12 kb longer than telomeres in wild type cells. This pattern of exceptionally long telomeres is similar to that seen in human cell lines and tumors that maintain telomeric DNA without expressing telomerase. The maintenance of these long telomeres requires the continuous presence of Rad52p. However, even in the presence of Rad52p, these long telomeres steadily shorten, suggesting that even in cells that maintain their telomeres by telomere-telomere recombination, recombinational mediated telomere lengthening is relatively rare.

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Shu-Chen Teng 8/23/99

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(4) TABLE OF CONTENTS

(5) INTRODUCTION

Telomeres, the protein-DNA structures found at the natural ends of eukaryotic chromosomes, are required to protect chromosomes from degradation and end-to-end fusion and to facilitate their complete replication. In most organisms, telomeric DNA consists of a short, tandemly repeated sequence that has clusters of G residues in the strand that runs 5'to 3' towards the chromosome end. For example, Saccharomyces chromosomes end in ~ 350 ± 75 bps of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA (figure 1A). In addition, many eukaryotes have middle repetitive DNA elements or telomere associated sequences immediately internal to the simple repeats. In *S. cerevisiae*, there are two such sequences, X and Y'. X is a heterogeneous sequence found at virtually all telomeres (Louis and Haber, 1992). Y' is found in one to four tandem copies, immediately internal to the $C_{1\text{-}3}A/TG_{1\text{-}3}$ repeats, on many, but not, all yeast telomeres (Chan and Tye, 1983; Zakian and Blanton, 1988). There are two classes of Y' elements, Y'-short and Y'-long, with the 5.3 kb Y' short differing from the 6.7 kb Y' long by a 1.4 kb internal deletion (Louis and Haber, 1992). When Y' is tandemly repeated, a given array consists of all Y'-long or all Y'-short elements (Louis and Haber, 1990).

In most eukaryotes, including yeast, telomere replication is carried out by a special reverse transcriptase, telomerase, that uses a small CA-rich stretch in its RNA component to template extension of the G-rich strand (reviewed in (Blackburn, 1992; Zakian, 1995)). The genes encoding the RNA (*TLC1*) (Singer and Gottschling, 1994) and protein catalytic subunit (*EST2*) (Counter, et al., 1997; Lingner, et al., 1997) of the *Saccharomyces* telomerase have been identified. Several additional genes including *EST1*, which encodes a telomerase RNA associated protein (Lin and Zakian, 1995), and *CDC13*, which encodes a protein that binds telomeres *in vivo* (Bourns, et al., 1998) are also required for telomerase replication *in vivo* (Lendvay, et al., 1996; Nugent, et al., 1996; Lingner, et al., 1997). When any of the genes that are essential for the telomerase pathway is deleted, telomere length gradually shortens, chromosome loss increases, and most cells die (see for example, (Lundblad and Szostak, 1989)).

Telomerase is not the only mechanism that can maintain telomeric DNA. In *Drosophila*, transposition of telomere specific retrotransposons is the major pathway for telomere maintenance (Biessmann and Mason, 1997). Both telomerase and transposition contribute to telomere maintenance in the green alga *Chlorella* (Higashiyama, et al., 1997). In some insects, such as the mosquito *Anopheles* (Roth, et al., 1997) and the dipteran *Chionomus* (Lopez, et al., 1996), telomere-telomere recombination is thought to be the sole mechanism for maintaining the repeats at chromosome ends.

Even in organisms that normally rely on telomerase, telomerase independent mechanisms of telomere maintenance exist. Although most cells in *S. cerevisiae* (Lundblad and Blackburn, 1993), *Schizosaccharomyces pombe* (Nakamura, et al., 1997), and *K. lactis* (McEachern and Blackburn, 1996) that lack the gene for a telomerase component die, survivors arise relatively frequently in all three organisms. In both *S. cerevisiae* and *K.lactis*, generation of survivors requires *RAD52* dependent recombination. In *S. cerevisiae* (discussed in more detail below), most survivors have very short telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ tracts but long tandem arrays of Y' DNA. In contrast, in *Kluveromyces lactis*, survivors have long tracts of telomeric repeats (McEachern and Blackburn, 1996). *S. pombe* can escape the telomerase requirement in two ways, by amplification of its telomere associated repeats (TAs), presumably by recombination, or by losing both TA and telomeric DNA, followed by end to end fusions to generate circular chromosomes (Nakamura, et al., 1998). As some human cell lines (Bryan, et al., 1995) and tumors (Bryan, et al., 1997) that lack telomerase have very long telomeres, telomerase bypass pathways exist in mammals as well.

The generation of survivors in the absence of telomerase has been studied most extensively in *est1Δ* strains of Saccharomyces (Lundblad and Blackburn, 1993). In this pioneering study, the authors described two types of telomerase independent survivors based on the pattern of restriction fragments produced after digestion with *Xho*I and concluded that both types, called type I and type II in this paper, arise by tandem duplication of part or all of the sub-telomeric Y' element. Similar survivors were observed in *tlc1*, *est2*, *est3* and *est4* strains but the structure of DNA in these strains has not been characterized in detail (Singer and Gottschling, 1994; Lendvay, et al., 1996).

I reinvestigated the structure of telomeric DNA in type II survivors arising in a *tlc1* strain. I found that RNA mediated transposition of Y' elements to chromosome ends occurred but its frequency was too low to support telomere maintenance in the absence of telomerase. Likewise, type II survivors did not arise as a result of chromosome circularization. However, type II survivors did not, as proposed (Lundblad and Blackburn, 1993), have tandem duplications of the distal end of Y'. Rather, type II survivors had very long

terminal tracts of $C_{1-3}A/TG_{1-3}$ DNA, with some telomeres being as much as 12 kb longer than telomeres in wild type cells. This pattern is similar to the exceptionally long telomeres in human tumors (Bryan, et al., 1997) or cultured cells (Bryan, et al., 1995) that lack telomerase. The maintenance of these elongated telomeres required Rad52p, but reintroduction of telomerase resulted in the very slow loss of telomeric DNA until all telomeres returned to wild type lengths.

(6) BODY

In my last annual report covering 1997-1998, I described the identification of a novel pathway for telomere formation. I did further characterization of this pathway in the last 12 months. Materials and methods, and the results are described as follows.

Materials and Methods

Plasmids, yeast strains, yeast transformations, genetic manipulation.

All the yeast operations were performed according to standard protocols (Rose, et al., 1990). Yeast strains used in this study were derivatives of YPH501 (*MATa/MATα ura3-52/ura3-52 lys2-801 amber/ lys2-801 amber ade2-101 ochre/ ade2-101 ochre trp1Δ63/trp1Δ63 his3Δ200/his3Δ200 leu2-Δ1/leu2-Δ1*) (Sikorski and Hieter, 1989). YPH501 *tlc1::LEU2/TLC1* was constructed by transforming *Xho*I digested pBlue61::*LEU2* (kindly provided by D. Gottschling) (Singer and Gottschling, 1994) into YPH501 and selecting Leu^+ transformants. To complement a *tlc1* strain, a *CEN* plasmid containing *TLC1* was made. The full-length *TLC1* gene plus 1 kb of 5'- and 0.5 kb of 3'-flanking sequences was cloned into pRS317, a vector having *LYS2* as a selectable marker (Sikorski and Hieter, 1989). pRS317*TLC1* was transformed into YPH501 *tlc1::LEU2/TLC1* and sporulated. YPH *tlc1::LEU2* segregants carrying pRS317*TLC1* were selected by on complete medium lacking leucine and lysine.

The *his3AI*-5', *URA3*, and *his3AI*-3' were amplified by PCR using, respectively, *his3AI*-5', 5' (GGACTAGTGCTGCAGCTTTAAATATCG) and *his3AI*-5', 3' (CCCGCTCGAGATGGTCCTCTAGTACACTC), *URA3*, 5' (CCCGCTCGAGCTTTTCAATTCAATTCATC) and *URA3*, 3' (CTCCCCGCGGGTAATAACTGATATAAT), and *his3AI*-3', 5' (CTCCCCGCGGTGTCACTACATAAGAAC) and *his3AI*-3', 3' (TGCTCTAGATGGTCCTCTAGTACTCTC) as primers (underlined segments indicate restriction sites) and pTy*his3AI* (Teng, et al., 1996) as a template. To make pSL300*his3AI-URA3-his3AI*, *Spe*I *Xho*I digested *his3AI*-5', *Xho*I *Sac*II digested *URA3*, and *Sac*II *Xba*I digested *his3AI*-3' PCR-amplified fragments were cloned sequentially into the multiple cloning sites of pSL300 (Brosius, 1992). The fragment for tagging the 3' untranslated region of the Y' elements with *his3AI-URA3-his3AI* was amplified by PCR using 50 bp Y' sequences that spanned the stop codon of the Y' ORF2 at the ends of the primers and pSL300*his3AI-URA3-his3AI* as a template. The resulting Y'-*his3AI-URA3-his3AI*-Y' PCR-amplified fragment was transformed into the YPH *tlc1::LEU2* strain carrying pRS317*TLC1*. Y'-*his3AI-URA3-his3AI* tagged strains were selected on medium lacking uracil. Cells that had lost the *URA3* gene by pop out recombination were selected on 5-fluoroorotic acid (5-FOA) (Boeke, et al., 1984). Tagging of individual telomeres by *his3AI* was confirmed by Southern blot analysis using Y', and *his3AI* probes as described below.

Formation of survivors.

To lose the pRS317*TLC1* plasmid, cells were grown on YEPD plates overnight and then replica plated to α-aminoadipate plates to identify Lys^- cells (Chattoo, et al., 1979). Single colonies were restreaked on α-aminoadipate plates. Colonies from α-aminoadipate plates were then streaked on YEPD plates for single colony purification. This procedure was repeated 5 times on YEPD plates to allow cellular senescence to occur and survivors to appear. Plates were incubated at 30°C for 3 days. Survivors first appeared after 4 restreaks on YEPD plates. Alternatively, survivors were obtained by innoculating single colonies from the α-aminoadipate plates into 10 ml YEPD medium, growing these to stationary phase by incubation at 30 °C for 3 days, and then diluting the cultures 1 to 10,000 into fresh YEPD medium. This procedure was repeated 3 or 4 times and then cells were plated on YEPD plates to identify survivor colonies.

DNA preparation, enzyme digestion, Southern blot analysis and gel electrophoresis.

Genomic DNA preparation and Southern blot analysis were performed as previously described (Monson, et al., 1997). S1 nuclease and mung bean nuclease treatments were performed according to manufacture instructions (New England BioLabs). Two-dimensional gel electrophoresis (Brewer and Fangman, 1987) and alkaline denaturing gel electrophoresis (Sambrook, et al., 1989) were performed as

described. For the *Bal*31 exonuclease digestion experiment, 70 µg of genomic DNA from wild-type or type II survivors was digested with 3 units of *Bal*31 (New England BioLabs) in a 100 µl final volume. 14 µl of digested DNAs were removed every ten minutes, subjected to phenol/chloroform extraction and ethanol precipitation, and digested with *Xho*I. The following probes were used for Southern hybridization: a 270 bp $C_{1\text{-}3}A$ fragment, a 1.5 kb *Sph*I–*Sal*I fragment from the 5' end of Y', a 4.2 kb *Sal*I-*Xho*I fragment from the middle region of Y', a 341 bp *Xho*I-*Kpn*I fragment of from the 3' end of Y', a 586 bp *Nde*I-*Nsi*I fragment of *HIS3*, a 1 kb 5'-*Eco*RI fragment of *PIF1* and a 350 bp PCR fragment of *TLC1*. Probes were random labeled using the RTS-Rad prime system (Life Technologies).

For pulse field gel electrophoresis, yeast chromosomal DNA blocks were prepared by mixing equal volumes of yeast cells from stationary phase cultures with 1% low melting agarose (FMC BioProducts) as described (Rose, et al., 1990). Pulse-field gel electrophoresis was performed using the contour-clamped homogeneous electric field-dynamically regulated CHEF-DR III system (BioRad). Chromosomes were separated on a 1% agarose gel in 0.5x TBE buffer at 14°C for 30 hours at 6.0 V/cm (200V) using a 120° included angle with a 60-120 second linear switch time ramp.

Inverse PCR, cloning and sequencing.

3 µg of genomic DNA from wild-type and two independently isolated type II survivors with *his3AI* tagged telomeres was digested with *Xho*I. The *Xho*I digested fragments were made blunt ended by using Klenow enzyme in the presence of all four nucleotides. Half of each reaction mixture was subjected to Southern blot analysis to determine the size of the *his3AI* tagged *Xho*I-digested telomere fragments. The other half of the reaction was separated on a 0.6% gel. DNAs in the correct size range to contain the *his3AI* tagged *Xho*I-digested telomere fragments were gel-purified and ligated at 14°C overnight in a total volume of 200 µl. PCR was carried out using 4 µl of the ligation mix. The PCR conditions were: 30-40 cycles of 30-second 94°C denaturation, 1-min 69°C annealing, and 3-min 72 °C extension. The primers were P1 (5'-TAGCGACCAGCCGGAATGCTTGG-3') and P2 (5'-ACGATGTTCCCTCCACCAAAGGTG-3') facing opposite to each other in *his3AI* (see figure 5). A further PCR amplification step was performed using nested primers P3 (5'-AGCGCTCGTCATGGAACGCAAAC-3') and P4 (5'-CGAGAGTAGAGGTAGATGTGAGAG-3') facing opposite to each other in the Y' element (see figure 5). The PCR generated products were cloned into the pCRIITOPO vector (Invitrogene) and transformed into *E. coli* STAB2 cells (GIBCO-BRL). Sequencing was performed using Cycle sequencing kits (Epicentre Technologies) with primers P3 and P4.

Results

Identification of survivors in a *tlc1* strain.

Although most *est1* cells die, telomerase independent survivors appear after ~50-100 generations (Lundblad and Blackburn, 1993). This previous study identified two types of survivors in an *est1* strain that are distinguishable by their pattern of telomeric *Xho*I fragments. There is a single site for *Xho*I in Y' (Fig. 1A). The majority (63%) of *est1* survivors (called type I survivors in this paper) have three major *Xho*I fragments that hybridize to the Y'3'probe (Fig. 1A), which detects the distal portion of Y'. The sizes of these bands are ~1.3 , 6.7, and 5.2 kb. The ~1.3 kb fragment is the terminal fragment from Y' telomeres and consists mainly of Y' DNA with a very short stretch of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA. The strong hybridization at 6.7 and 5.2 kb is due to, respectively, tandemly repeated Y' long and Y' short elements (Fig.1A). In contrast, *Xho*I digestion of DNA from type II survivors yields many differently sized *Xho*I fragments that hybridize to both $C_{1\text{-}3}A/TG_{1\text{-}3}$ and 3' Y' probes but not to probes from other regions of Y' (Lundblad and Blackburn, 1993). The pattern of telomeric *Xho*I fragments varies among independent type II survivors. The authors concluded that telomeres of type II survivors have tandem duplications of the distal segment of Y'(Lundblad and Blackburn, 1993).

To further understand telomerase-independent mechanisms for telomere maintenance in *S. cerevisiae*, a strain lacking *TLC1*, the gene encoding the RNA component of telomerase was created. This strain contained a *LYS2* CEN plasmid harboring the wild-type *TLC1* gene to complement the chromosomal *tlc1* deletion. Cells that lost the *TLC1* plasmid were identified and then restreaked multiple times to obtain survivors. Although most *tlc1* cells died, after ~100-125 generations, faster growing survivor cells appeared. Genomic DNA from twenty-four independent survivors, as well as from wild type and early passage *tlc1* strains was isolated, digested with the *Xho*I restriction enzyme, and analyzed by Southern blot analysis using a $C_{1\text{-}3}A/TG_{1\text{-}3}$ probe. Most (21 of 24) survivors were type I (two examples are shown in Fig. 1B, lanes 3 & 4) and three were type II (Fig. 1B, lanes 5 & 6). Additional type II survivors were obtained in independent experiments.

The growth characteristics of nine type I and nine type II survivors were analyzed by restreaking each survivor ten times on YEPD plates. The growth rate of type II survivors and wild-type colonies was very similar whereas type I survivors grew more slowly and their growth rate fluctuated in different restreaks, with senescing cells reappearing at different times during outgrowth. Type I and type II survivors from an *est1* strain had similar growth properties (Lundblad and Blackburn, 1993). I conclude that the survivors obtained in a *tlc1* strain were indistinguishable from those obtained in an *est1* strain.

Survivors are not generated in a *rad52 est1* strain (Lundblad and Blackburn, 1993) nor in a *rad52 tlc1* strain (S.-C.T and VAZ, unpublished results.) I also isolated type II survivors in a *rad52Δ tlc1* strain carrying a *RAD52* plasmid. After survivors were generated, cells that had lost the *RAD52* plasmid were identified. The *tlc1 rad52* type II survivors formed heterogeneous colonies similar to those seen in senescent *tlc1* cells, and were unable to grow after 3 to 4 restreaks. Thus, *RAD52* function was needed continuously to maintain type II survivors.

Telomeres of *tlc1* survivors are not maintained through transposition.

As it seemed unlikely to us that recombination would generate tandem duplication of just the 3' end of Y', I considered that type II survivors might be generated by a different mechanism. *Drosophilia* telomeres consist of retrotransposons (Higashiyama, et al., 1997) (Biessman, et al., 1990). Y' elements are 5 to 7 kb in length, contain two open reading frames (ORFs) in different overlapping reading frames of the same direction, and are flanked by TG_{1-3} repeats (Louis and Haber, 1992), features characteristic of many retrotransposons (Eickbush, 1992). Moreover, many integrated copies of retrotransposons have truncated 5' ends due to premature termination of reverse transcription (Eickbush, 1992), a situation that could explain the proposed tandem duplication of just the 3' end of Y'. In yeast, a chromosome without a telomere is seen as a double strand break (Sandell and Zakian, 1993), and retrotransposons are able to repair chromosomal breaks (Teng, et al., 1996). The *RAD52* dependence for generating survivors would be explained if Y' cDNAs were added to chromosome ends by homologous recombination. These considerations led us to test if type II survivors are generated by RNA mediated transposition of Y' DNA.

To test this possibility, I first tagged the 3' end of Y' elements with the *his3AI* marker (Curcio and Garfinkel, 1991) (Fig. 2). The *his3AI* gene is designed to detect reverse transcription mediated events in yeast. For example, this marker was used to demonstrate retrotransposon reverse transcriptase-mediated chromosomal break repair (Teng, et al., 1996). In *his3AI*, the *HIS3* ORF is interrupted by a 104 bp artificial intron (AI) that is oriented opposite to the direction of *HIS3* transcription. Transcription of the *his3AI* gene results in a nonsplicable RNA. The *his3AI* gene was inserted within Y' such that transcription from the Y' promoter generates a transcript containing antisense *HIS3* sequences interrupted by the AI intron in a spliceable orientation. Reverse transcription of the spliced RNA, followed by either recombination or transposition of the *HIS3* cDNA will generate His^+ colonies.

Both wild-type and *tlc1* strains that contained *TLC1* on a plasmid were transformed with a construct having the *his3AI* marker inserted into the middle of the 0.9 kb 3' end of Y' DNA at the downstream boundary of ORF2 (Fig. 2). Transformants were screened by Southern blotting to obtain strains that had a single *his3AI* tagged telomere. Since different telomeres have zero to four copies of Y', the *his3AI* marker could insert either within an internal or a terminal Y' element. I recovered eight wild-type strains of which three had the *his3AI* gene inserted within an internal Y' and five within a terminal Y'. I recovered fourteen *tlc1* strains containing the *TLC1* plasmid, four tagged at an internal and 10 at a terminal Y' element.

I selected for loss of the *TLC1* plasmid in *tlc1* cells containing a *his3AI* tagged telomere using the liquid assay described in the methods. The liquid culture scheme favored isolation of type II survivors because of their growth advantage compared to type I survivors. Indeed, Southern blot analysis revealed that most (24 of 24) survivors had the *Xho*I restriction pattern diagnostic for type II survivors. Cultures were then plated onto YEPD plates to determine total cell number and complete plates lacking histidine to determine the fraction of His^+ cells. If type II survivors are generated via a cDNA intermediate, the majority of them should have a His^+ phenotype. Three *tlc1* His^+ colonies were recovered from a total of 4.2×10^9 post-senescent *tlc1* cells. No His^+ colonies were identified in 6.6×10^9 wild-type cells. Although all three His^+ colonies from the *tlc1* survivors had the structure expected for cDNA mediated movement of the *his3AI* gene to a chromosome end, these events were too rare to provide a general explanation for the formation of type II survivors.

Terminal but not internal Y' elements are altered during generation of Type II survivors.

I utilized strains having a single *his3AI* tagged telomere to determine the structure of telomeric DNA in type II survivors. Twelve independent *tlc1* strains (strains 1 - 12) each with a single *his3AI* tagged

Y' element were generated. Three of the twelve strains (strains 1, 2 and 6) had *his3AI* at an internal Y'. Strain 1 was marked at an internal Y'-short element and strains 2 and 6 were marked at an internal Y'-long element. The nine other *tlc1* strains had *his3AI* inserted within a terminal Y' element. Each of the twelve strains was diluted into liquid YEPD medium to generate survivors, a method that favors type II survivors due to their faster growth rate. Two independent survivors (a and b) from each of the twelve strains were examined in detail.

Genomic DNA was prepared from each of the 24 survivors, digested with *Xho*I, and examined by Southern blotting using probes for telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA (Fig. 3A), the 3' end of Y' (Fig. 3B), the 5' and middle parts of Y', and *his3AI* (Fig. 3C). The telomeric and 3' Y' probes detected multiple irregular sized bands in each of the 24 strains that ranged in size from ~1.5 to 10 kb. Most *Xho*I fragments hybridized to both the telomeric and 3' Y' probe (compare panels A and B) but not to the 5' or middle regions of Y'. This pattern is characteristic of type II survivors (Lundblad and Blackburn, 1993). Thus, 24 of 24 recovered survivors were type II survivors. Given the growth advantage of type II over type I survivors and the method used to generate survivors, the predominance of type II survivors was expected.

To determine the fate of individual telomeres during survivor formation, the same blot was hybridized with a *his3AI* probe (Fig. 3C). The sizes of the *his3AI* hybridizing sequences in survivors obtained from *tlc1* strains with *his3AI* inserted into an internal Y' (strains 1, 2 and 6) were unchanged compared to the starting *tlc1* strain. These bands had the size expected for insertion into Y' short (strain 1, a and b) or the size expected for insertion into Y' long (strains 2 and 6, a and b). These data suggest that during formation of type II survivors, internal Y' elements were not subjected to major rearrangements. In contrast to the similarity of the structure of the *his3AI* gene in independent survivors when *his3AI* was inserted within an internal Y', the pattern of $C_{1\text{-}3}A/TG_{1\text{-}3}$ (Fig. 3A) or 3' Y' (Fig. 3B) hybridization was different in the two independent survivors from each of the three strains.

The pattern of *his3A*I hybridization in the 21 survivors obtained from *tlc1* strains that had *his3AI* inserted at a terminal Y' was diverse. Of the 21 survivors, 18 still had a single *his3AI* tagged telomere, one had no tagged telomere (11a), and five had two or more *his3AI* tags (strains 9a, 10 a and b, and 12 a and b). In no case, did the two survivors obtained from the same starting *tlc1* strain have the same structure. The only consistent feature was that the tagged Y' in the survivor was almost always larger than the tagged Y' fragment in the parental *tlc1* strain (position of parent band marked terminal Y'-*his3AI* in Fig. 3C). These data indicate that the structure of terminal Y' elements is usually altered during the formation of a type II survivor.

To obtain a better understanding of the kinds of changes that can occur during formation of type II survivors, I used pulse field gel (PFG) electrophoresis to determine the chromosomal location of the *his3A*I tag in four independent type II survivors as well as in their parent strains (Fig. 3D). In strain 3, there was a single copy of *his3AI* inserted within a terminal Y' element on either chromosome VII or XV (these chromosomes co-migrate in PFG). In this case, the telomere lengthening that accompanied transition to a type II survivor did not involve movement of *his3AI* to a different chromosome, as the position of *his3AI* in the two survivors was the same as the starting strain (Fig. 3D). Strain 10 also had a single copy of *his3AI* within a terminal Y' element, this time on chromosome VI. Both strain 10 survivors retained *his3AI* on chromosome VI but in addition had *his3AI* on other chromosomes (two new copies of *his3AI* in survivor 10a, on chromosome VII and chromosome IV or XII; one new copy in survivor 10b, on chromosome IV or XII). Digestion with *Sal*I can be used to determine if *his3AI* was inserted into Y' short or a Y' long (Fig. 1A). In strain 3 and its two survivors, *his3AI* was embedded in Y' long (Fig. 3E). However, during generation of survivors 10a and 10b, the *his3AI* tag moved not only to a new chromosome but from a Y' short to a Y' long element (Fig. 3E). These results indicate that both intra- and inter-chromosomal recombination occur during generation of type II survivors.

The *his3AI* sequences are still near the physical end of the chromosome in type II survivors. As the *his3AI* sequences inserted within a terminal Y' element were rearranged during formation of type II survivors, it is possible that they were no longer at the ends of linear chromosomes. For example, chromosome ends without a telomere could fuse to form a circular chromosome as seen in telomerase-minus *S. pombe* (Nakamura, et al., 1998). If the *his3AI* marker were at the physical end of a chromosome in type II survivors, it would be sensitive to digestion by the exonuclease *Bal*31. To address this possibility, genomic DNA was prepared from a wild-type strain with *his3AI* inserted within a terminal Y' (Fig. 4A, left) and four independent type II survivors (Analysis of survivor 3b is shown in Fig. 4A, right). DNA was digested with *Bal*31 with samples removed at 10 minute intervals. The DNA was then digested with *Xho*I, subjected to electrophoresis and analyzed by hybridization to a *his3AI* probe. As the *his3AI* hybridizing

sequences shortened at the same rate in both wild type and type II survivor 3b DNA, *his3AI* sequences were near a free end in the 3b survivor. However, it took longer to degrade the *his3AI* sequences in the 3b survivor DNA than in its parent strain, indicating that in the type II survivor, the *his3AI* sequences were further from the physical end of the chromosome. Hybridization of the same blot with the internal *PIF1* probe demonstrated that non-telomeric sequences were not *BAL*31 sensitive (Fig. 4A). Similar results were obtained with three other type II survivors. These data argue that the rearrangement of *his3AI* sequences that accompanied generation of type II survivors did not alter the telomere proximal location of the tag.

The *his3AI* sequences are still embedded in Y' DNA in type II survivors and have a structure very similar to that of the starting strain.

The *Bal31* data suggest that the *his3AI* sequences in a type II survivor were on a terminal *XhoI* fragment but were further from the chromosome end than in the parental *tlc1* strain. Restriction enzyme analysis was used to demonstrate that the DNA proximal to the *his3A* gene in different type II survivors had the characteristics expected for Y' DNA. Genomic DNA from each of four survivors (3a, 3b, 10a and 10b in figure 3C) as well as DNA from their parent strain (3 and 10) was digested with five different restriction enzymes. The enzymes, *Sal*I, *Hind*III, *Eco*RI, *Bgl*II, and *Kpn*I, all have recognition sites within Y'and/or *his3AI* DNA (see Fig. 1A for location of sites). Digested DNA was analyzed by Southern blotting using a *his3AI* probe. The data using *Eco*RI are shown in Fig. 4B. The *Xho*I fragments that hybridized to the *his3AI* probe were larger in each type II survivor than the 2.1 kb *Xho*I fragment produced in the parent *tlc1* strains (Fig. 4B). For example, in survivor 3b, a 3.5 kb *Xho*I fragment hybridized to *his3AI* whereas in survivor 3a, a 2.4 kb *Xho*I fragment hybridized to *his3AI* (Fig. 4B). There is an *Eco*RI site in the Y' element 2.6 kb internal to the *Xho*I site (Fig. 1A). If *his3AI* were still embedded in the same place in Y' in survivor strains, *his3AI* should hybridize to a 6.1 kb *Eco*RI fragment in survivor 3b (3.5 + 2.6) and a 5 kb *Eco*RI fragment in survivor 3a (2.4 +2.6), exactly the pattern seen (Fig. 4B). Likewise, in survivors 10a and b, the *Eco*RI fragment(s) that hybridized to *his3A*I were 2.6 kb larger than the *his3AI* hybridizing *Xho*I fragments. As this result was obtained with five of five restriction enzymes, the *his3AI* fragments in type II survivors behaved as if they were embedded within Y' DNA.

Newly elongated telomeric fragments in type II survivors are $C_{1\text{-}3}A/TG_{1\text{-}3}$ tracts.

An inverse PCR strategy (Ochman, et al., 1988) (Fig. 5) was used to obtain the sequence of the DNA to either side of *his3AI* in two type II survivors, 3a and 3b, as well as in the parental *tlc1* strain (Fig. 5). Genomic DNA from each of the three strains was digested with *Xho*I and the restriction fragments rendered blunt ended by treatment with the Klenow fragment of DNA polymerase I. The *his3AI Xho*I fragments from the parent (2.1 kb), survivor 3a (2.4 kb) and survivor 3b (3.5 kb) strains were gel-purified, and treated with ligase. Because the DNA was dilute, ligation generated intramolecular circles. The circularized *Xho*I fragments were subjected to PCR amplification using primers P1 and P2, which are 395 bps apart and facing in opposite directions within the *his3AI* gene (Fig. 5). For each strain, the expected sized PCR product was obtained but only in ligase treated DNA. Since there were other ligase-independent PCR products in the first PCR amplification, I did an additional PCR amplification on the products of the first reaction using nested primers P3 and P4. P3 annealed at the very 3' end of Y', just upstream of the telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ telomeric tract. P4 annealed just downstream of the *Xho*I site in Y' and was oriented towards the *Xho*I site (see Fig. 5). Again, PCR fragments of the appropriate size were obtained. These PCR products hybridized to a $C_{1\text{-}3}A/TG1_{\text{-}3}$ probe. Because the PCR products are from a population of DNA molecules, the exact sequence of telomeric DNA will vary from molecule to molecule (while still conforming to the $C_{2\text{-}3}A(CA)_{1\text{-}6}/(TG)_{1\text{-}6}TG_{2\text{-}3}$ consensus (Wang and Zakian, 1990)). Although I could not obtain a precise telomeric sequence from PCR amplified DNA, when the pool of molecules was sequenced using the P3 primer, the products from all three strains consisted only of T and G residues. Using the P4 primer, 45 bps of Y' sequence was followed by DNA consisting of only A and C residues.

To determine the sequence of individual telomeres, I cloned the products from the second PCR reaction from survivor 3a and from its parent *tlc1* strain prior to its losing pRS317-*TLC1* into vector pCRIITOPO (Invitrogene). Whereas telomeres from the parent strain were easily recovered, type II survivor telomeres were only clonable in STAB2 *E. coli* (GIBCO-BRL), a strain used to stabilize long tracts of repetitive DNA. I obtained an insert of 0.6 kb, the appropriate size for the telomeric *his3AI* fragment from survivor 3a. Using the P3 and P4 primers, I sequenced ~100-200 bps from each end of the insert and found that it consisted of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA. Thus, the 100-200 immediately distal to Y' and the 100-200 bps from the very end of the *his3AI* tagged telomere consisted of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA.

The sequencing data suggest that all of the DNA distal of Y' in type II survivors consisted of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA, leading to the conclusion that individual telomeres had very long and variable lengths of

telomeric DNA. Alternatively, the slow mobility of these telomeric fragments might be explained by their having an unusual structure that affected their mobility in agarose gels. For example, type II survivors might have telomeres with very long single-stranded TG_{1-3} tails as seen in *cdc13-1* cells at semi-permissive temperatures (Garvik, et al., 1995), and these single-stranded tails might form secondary structures that reduced fragment mobility. However, the mobility of *his3AI* tagged telomeres did not change after treatment with the single strand specific S1 or mung bean nucleases. Moreover, analysis by both two-dimensional (Brewer and Fangman, 1987) and alkaline denaturing (Sambrook, et al., 1989) gel electrophoresis revealed no difference between telomeres of type II survivors and wild-type cells. Thus, the slower migration of *his3AI* tagged telomeres in type II survivors is surely due to their being longer, not to their having an unusual structure.

The DNA distal of *his3AI* in type II survivors lacks recognition sites for four different enzymes with 4 bp recognition sites, suggesting that it consists solely of $C_{1-3}A/TG_{1-3}$ DNA.

The cloning and sequencing results confirmed that the *his3AI* marker was still embedded at the same site within Y'DNA as in the starting strain and that there was at least 200 bps of $C_{1-3}A/TG_{1-3}$ DNA distal to Y'. If the entire telomeric tract consisted of $C_{1-3}A/TG_{1-3}$ DNA, the tract would lack sites for most restriction enzymes, even enzymes that cut frequently in yeast DNA. Alternatively, if the $C_{1-3}A/TG_{1-3}$ tract were interrupted by non-telomeric DNA, it would be subject to digestion by such enzymes. To test this possibility, genomic DNA from a wild type strain (Fig. 6, wt), a type I survivor (lane marked I; Fig. 6), and ten independent type II survivors (lanes marked II; Fig. 6) was digested with a mixture of *Alu*I, *Hae*III, *Hin*fI and *Msp*I restriction enzymes. Each of these enzymes recognizes a different four-base pair sequence and together they are expected to reduce yeast DNA to, on average, 96 bp. There are many sites for these enzymes within Y', including sites 358 bp downstream of the 5' end of Y' and 42 bp upstream of the 3' end of Y'. The DNA was analyzed by Southern blotting using a $C_{1-3}A/TG_{1-3}$ probe.

As the wild type strain used for this study had telomeres of ~375±75 bps, digestion with the four enzymes is expected to generate $C_{1-3}A/TG_{1-3}$ hybridizing fragments of ~375 + 42 bp fragments from Y' bearing telomeres, ~0.5 kb fragments from tandem Y' elements, and fragments of up to 1.1 kb from X telomeres. Consistent with this expectation, digestion of DNA from wild type cells with the four enzymes released $C_{1-3}A/TG_{1-3}$ fragments that were mostly smaller than 1 kb (Fig. 6). DNA from the type I survivor yielded very short fragments, ~300 bps. In contrast, fragments containing $C_{1-3}A/TG_{1-3}$ DNA in type II survivors were large, ranging up to 12 kb. Hybridization of the same blot with the internal *TLC1* probe demonstrated that each sample was loaded with similar amounts of DNAs (Fig. 6). The fact that these large fragments hybridized intensely to the $C_{1-3}A/TG_{1-3}$ probe provided additional evidence that they consisted solely of $C_{1-3}A/TG_{1-3}$ DNA. I conclude that individual telomeres in type II survivors bear very long and variable length tracts of $C_{1-3}A/TG_{1-3}$ DNA.

Type II survivors are stable over time but their telomeres continuously shorten.

To test whether or not telomere structure in type I and type II survivors was stable, three independent type I survivors and 20 independent type II survivors were restreaked ten times on YEPD plates. DNA was prepared from different restreaks, digested with *Xho*I and analyzed by Southern blotting using a $C_{1-3}A/TG_{1-3}$ probe (Fig. 7A). Two of the type I survivors maintained a type I telomeric pattern throughout the restreaking period while the third type I survivor converted to a type II telomeric pattern between the first and fourth restreak (Fig. 7A, left panel). Concomitant with this switch, the growth rate of this survivor improved and became similar to that of other type II survivors. Thus, type I survivors can convert to a type II pattern during outgrowth.

In contrast, all 20 type II survivors retained the *Xho*I pattern of variable length telomeric fragments that is characteristic of type II survivors for ~250 cell divisions (~25 cell divisions per restreak) (see Fig. 7A, right panel for two examples). Although the general pattern of telomeric *Xho*I fragments did not change during sub-culturing, most of the $C_{1-3}A/TG_{1-3}$ hybridizing *Xho*I fragments in type II survivors appeared to shorten slowly over time (Fig. 7A). This shortening was especially apparent when these blots were reprobed with *his3AI* (Fig. 7B) which detects a single telomere. The rate of telomere shortening in type II survivors was 2.89 bps/cell division. The *his3AI* marker at the internal Y' of the second type II survivor was lost between fourth and seventh restreaks. These data suggest that even although type II telomeres are exceptionally long, they are nonetheless subject to continuous shortening and gene conversion as are wild type telomeres in a cell that has just lost *TLC1*.

Normal telomere length regulation is restored in type II survivors when *TLC1* is reintroduced.

As most cells that lack telomerase do not form survivors, generation of survivors might require a second event that activates a telomerase independent telomere maintenance pathway. If this model were

true, reintroduction of telomerase might not be sufficient to restore a wild type pattern of telomere structure. However, telomeres of type I survivors returned to wild-type lengths when the *EST1* gene was reintroduced in (Lundblad and Blackburn, 1993)). To determine if the telomere lengthening that occurs in type II survivors was reversible, I introduced a plasmid borne *TLC1* gene into both type I and type II survivors. Transformants were restreaked multiple times, and DNA prepared for Southern analysis after variable number of cell divisions in the presence of telomerase. DNA was digested with both *Xho*I (Fig. 7C) and multiple four base pair cutters (Fig. 7D). Telomeres of type I survivors reverted to wild type lengths very early after reintroduction of *TLC1* whereas it took many generations for type II telomeres to return to wild type lengths (19 restreaks or ~475 cell divisions). Similar results were obtained with three other type II survivors. The rate at which type II telomeres returned to wild type length was in average ~4.0 bps/cell generation with various rate within each generation. These data suggest that generation of type II survivors does not require mutations of genes involved in telomere length regulation.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Telomeres of telomerase-minus survivors are not maintained through transposition in yeast.
- Terminal but not internal Y' elements are altered during generation of survivors.
- The *his3AI* sequences are still near the physical end of the chromosome in type II survivors, are embedded in Y' DNA and have a structure very similar to that of the starting strain.
- Newly elongated telomeric fragments in type II survivors are $C_{1\text{-}3}A/TG_{1\text{-}3}$ tracts.
- Type II survivors are stable over time but their telomeres continuously shorten.
- Normal telomere length regulation is restored in type II survivors when *TLC1* is reintroduced.

(8) REPORTABLE OUTCOMES

a. This work was submitted to Journal "*Molecular and Cellular Biology*".
b. I talked this work at Gordon Conference, Plasmid and Chromosomal Dynamics section in August, 1999.

(9) CONCLUSIONS

Here I present evidence that *S. cerevisiae* can use both $TG_{1\text{-}3}$ tract (type II) and Y' element (type I) as substrates to extend their telomeres in cell lacking telomerase. I found that type II survivors replicate and maintain telomeres through *RAD52*-dependent homologous recombination. Sequencing results and Southern blot analysis showed that the long and irregularly extended telomeric fragments of type II survivors were all $TG_{1\text{-}3}$ sequences. These results dispute previous finding that type II survivors are generated by amplification of the 3' end of Y' (Lundblad and Blackburn, 1993). The telomeric *Xho*I fragment of Y' can always be hybridized by the 3' end of Y' probe even if only one copy of the 3' end of Y' in this fragment. In addition to the $TG_{1\text{-}3}$-$TG_{1\text{-}3}$ recombination, inter-chromosomal recombination and recombination between two different types of Y's were observed in type II survivors (figure 3D and 3E). Recombination between Y'-long and Y'-short is an interesting finding since previous observation showed that Y' recombined preferentially within its own subset (Louis and Haber, 1990). My results indicate that in telomerase-minus cells, *S. cerevisiae* replicates its telomeres through homologous recombination using either telomeric or sub-telomeric repeats as substrates. This bypass pathway for type II-like amplification appears to be very efficient. My result also demonstrates that type I-like Y' amplification is inefficient, and can revert to type II amplification. This explains why I saw amplification of Y' elements, and inter-Y's recombination in some type II survivors.

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(11) APPENDICES

Figures are attached and their numbers are indicated.

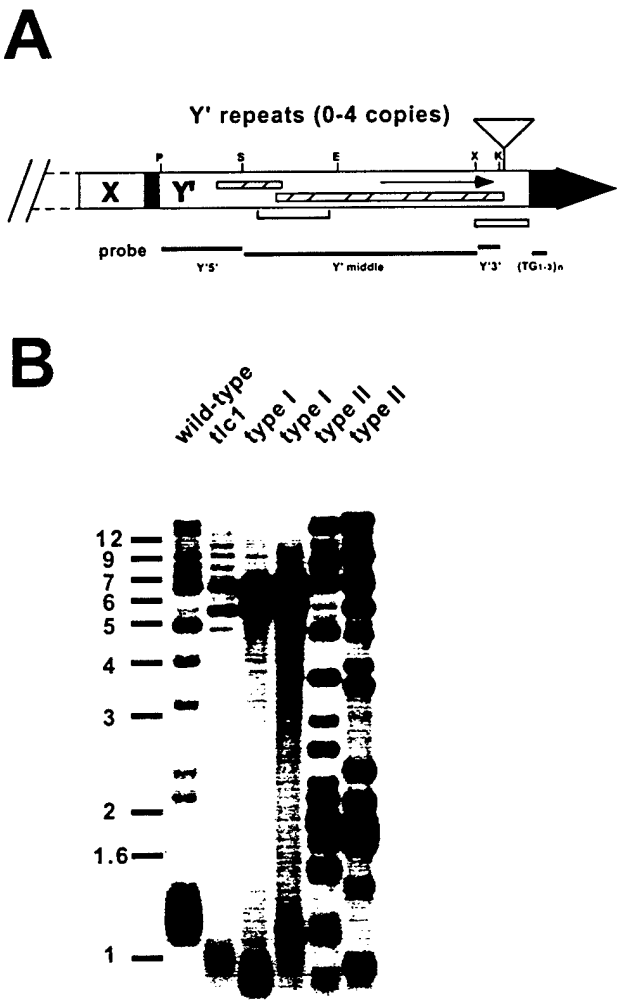

Figure 1. (A). Telomeric and sub-telomeric structure of *S. cerevisiae*. $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA is in black. The Y'-long element is between a ~50-100 bps internal stretch of $C_{1\text{-}3}A/TG_{1\text{-}3}$ DNA (26, 46) and a 300-400 bp terminal $C_{1\text{-}3}A/TG_{1\text{-}3}$ tract. The two open reading frames in Y' are shown as striped boxes and their direction of transcription indicated by the arrow. The position of the 0.9 kb *his3AI* insert is shown as a triangle. The deleted region in Y'-short is indicated by brackets (25). The 3' end of Y' is shown as an open rectangle. The solid lines indicate the restriction fragments used as probes. Restriction sites used for Southern blot analysis are as follows: P, *Sph*I; S, *Sal*I; E, *Eco*RI; X, *Xho*I; K, *Kpn*I. (B). Telomeres of *tlc1* survivors show two distinct patterns. Genomic DNA from wild-type, senescing *tlc1* cells, two independent type I *tlc1* survivors, and two independent type II *tlc1* survivors was digested with *Xho*I, fractionated through 1% agarose, and analyzed by Southern blotting using a $C_{1\text{-}3}A/TG_{1\text{-}3}$ probe. Size markers are in kb.

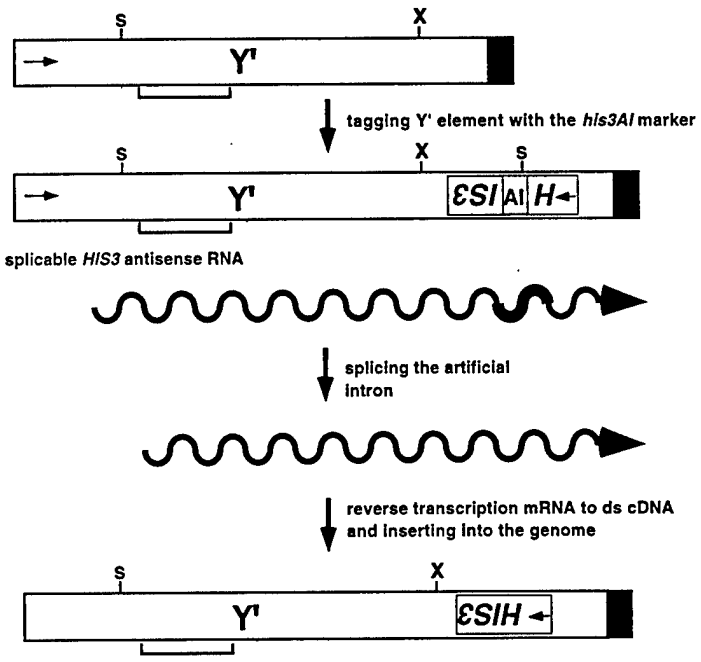

Figure 2. The *his3AI* gene (13) can be used to detect reverse transcriptase mediated addition of Y' to chromosome ends. The *his3AI* gene was inserted within the 3' end of Y' (see Fig. 1A). The *his3AI* gene is transcribed from the Y' promoter to generate an anti-sense *HIS3* transcript having the *AI* intron in a spliceable orientation. If this transcript is spliced and reverse transcribed, it will generate a cDNA containing an uninterrupted *HIS3* gene flanked on either side by Y' sequences. This Y' DNA can direct recombination of the cDNA to a chromosome end. *HIS3* gene expression is detected by growth on plates lacking histidine. The deleted region in Y'-short is denoted by brackets. Restriction sites used for Southern blot analysis are indicated: S, *Sal*I; X, *Xho*I.

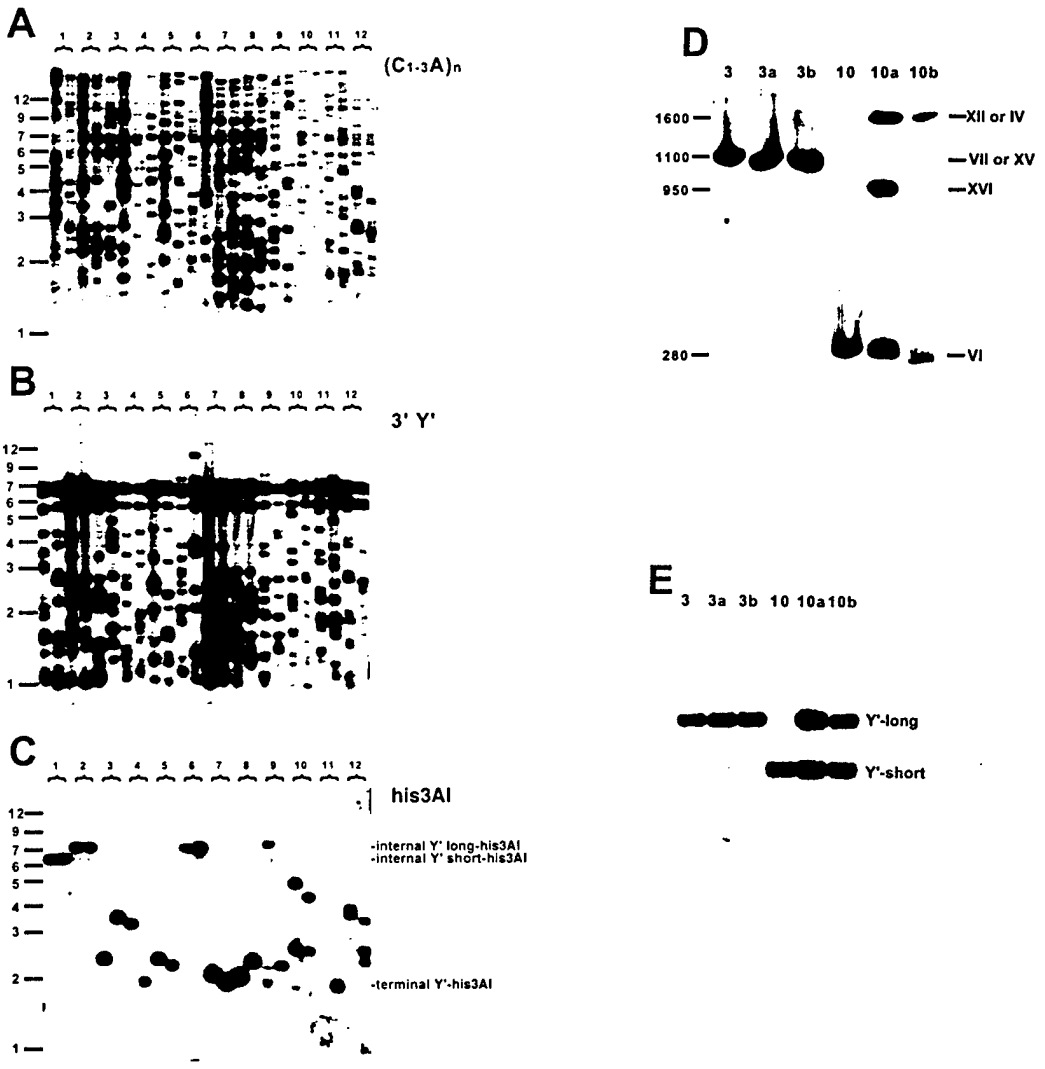

Figure 3. Southern blot analysis of telomeric DNA in independent type II survivors. Two *tlc1* survivors (named a and b) from each of 12 independent *tlc1* strains (number 1 – 12) with a single *his3AI* tagged Y' element were collected. Genomic DNA from each was digested with *Xho*I, fractionated through a 1% agarose gel, and analyzed by Southern blotting. The filter was hybridized sequentially with a (A) $C_{1\text{-}3}A/TG_{1\text{-}3}$, (B) 3' Y', or (C) *his3AI* probe. Most fragments smaller than 5.2 kb that hybridized to the $C_{1\text{-}3}A/TG_{1\text{-}3}$ probe (A) also hybridized to the 3' Y'probe. Fragments in A that did not hybridize to the Y' probe are marked with asterisks. Size markers are in kb. (D) Inter-chromosomal recombination in telomerase-minus *S. cerevisiae*. Chromosomes from two sets of type II survivors (3a, 3b, 10a and 10b) and their parent strains (3 and 10) were separated by pulse field gel electrophoresis. Size markers (kb) are shown at the left. Chromosome numbers are shown at the right (9). (E). Inter-Y' recombination in telomerase-minus *S. cerevisiae*. Genomic DNAs from the same sets of samples in D were digested with *Sal*I. After transfer to a nylon filter, the filters in (D) and (E) were hybridized with the *his3AI* probe.

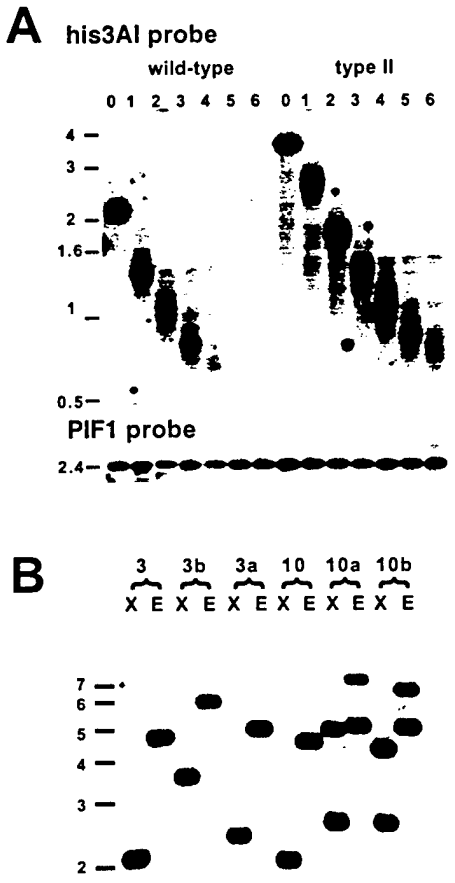

Figure 4. The *his3AI* gene remains near a free chromosome end and embedded within Y' DNA in type II survivors. (A) Genomic DNA from wild-type and a type II survivor (survivor 3b in figure 3C) was digested with *Bal*31 exonuclease for increasing amounts of time. Samples were removed at ten minute intervals, subjected to phenol/chloroform extraction and ethanol precipitation, and digested with *Xho*I. Digested DNAs were fractionated in a 0.7% agarose gel and analyzed by Southern hybridization using, sequentially, a *his3AI* (top) and internal probe (the *PIF1* gene). (B) Genomic DNA from two sets of type II survivors (3b, 3a, 10a, and 10b) and their parent strains (3 and 10) were digested with *Xho*I (X) and *Eco*RI (E) separately. Digested DNA was fractionated in a 0.7% agarose gel and analyzed by Southern hybridization using a *his3AI* probe. Size markers are in kb.

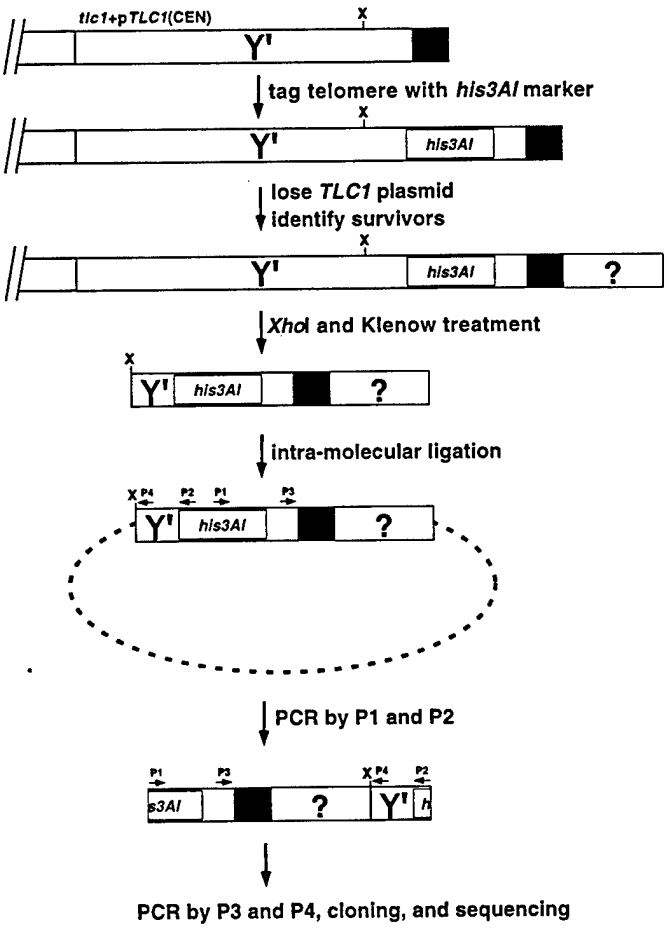

Figure 5. Inverse PCR strategy to determine the sequence of DNA flanking *his3AI* gene in type II survivors. Wild-type and *tlc1* strains with *TLC1* on a plasmid and containing a single *his3AI* tag were identified and then allowed to lose the *TLC1* plasmid. The black box represents the telomeric $C_{1\text{-}3}A/TG_{1\text{-}3}$ tract present before survivors were formed. The DNA added to the *his3AI* gene during formation of type II survivors is indicated by the question mark. Genomic DNA from wild-type and type II survivors was digested with *Xho*I, made blunt ended by treatment with Klenow enzyme in the presence of all four nucleotides. The *his3AI* tagged *Xho*I-digested telomere fragments were gel-purified and intramolecularly ligated. Ligated telomeric fragments were subjected to PCR amplification using primers P1 and P2. The P1-P2 PCR products were then subjected to nested PCR amplification using primers P3 and P4. Nested PCR products were further analyzed by cloning and sequencing.

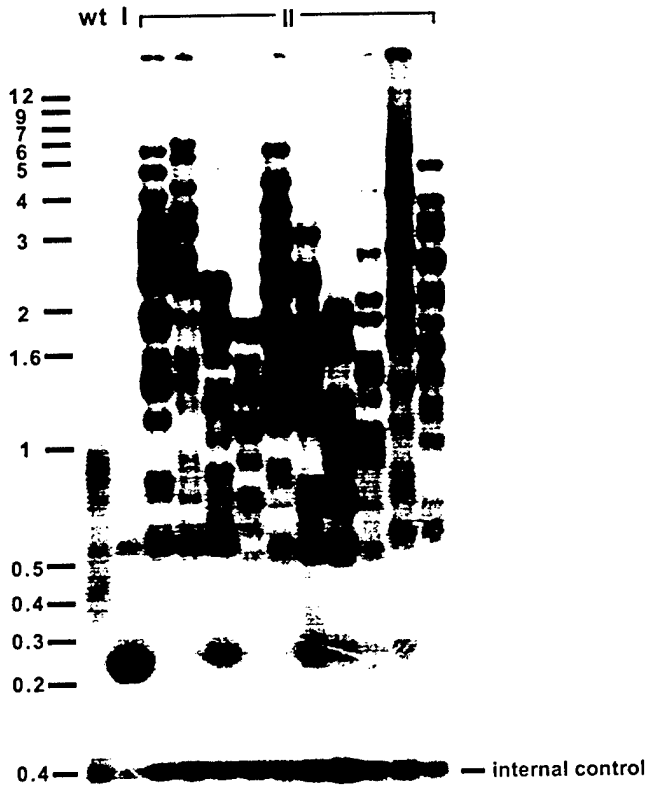

Figure 6. Newly elongated telomeric fragments in type II survivors are $C_{1\text{-}3}A/TG_{1\text{-}3}$ tracts. Genomic DNA from a wild-type strain, a type I survivor and ten type II survivors was digested with a combination of *Alu*I, *Hae*III, *Hin*fI and *Msp*I, which cut at the AGCT, GGCC, GANTC, and CCGG sequences, respectively, fractionated through 1.3% agarose, and transferred to a nylon filter. The filter was hybridized sequentially to a $C_{1\text{-}3}A/TG_{1\text{-}3}$ probe (top) and a probe for a non-telomeric fragment (taken from a region of *TLC1* that was not deleted; serves as a control for DNA loading). Size markers (kb) are shown at the left.

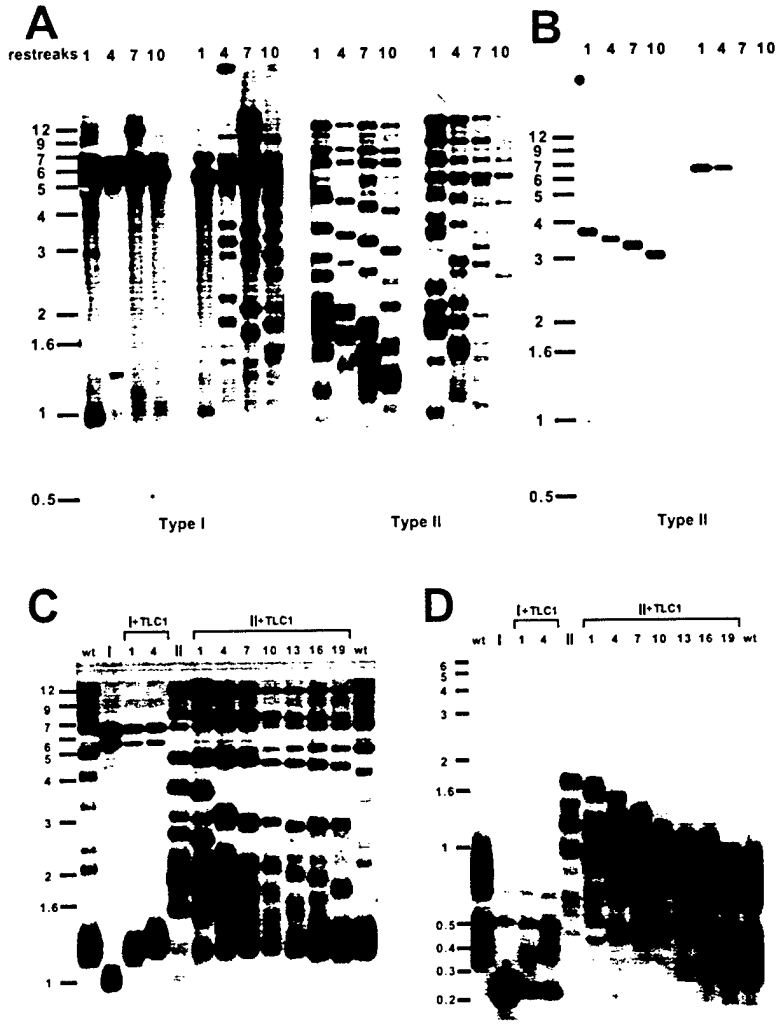

Figure 7. Telomeric changes in type I and type II survivors during outgrowth and after introduction of telomerase. (A) Stability of telomeric structure in type I and type II survivors. Two type I and two type II survivors were restreaked ten times on YEPD plates. Genomic DNA from the first, fourth, seventh and tenth restreak was digested with *Xho*I. Southern blot analysis was performed as in figure 1B. (B) Telomeres of type II survivors are subject to continuous shortening and gene conversion. The blot shown in the right panel of Fig. 7A was stripped and rehybridized with a *his3AI* probe. The *his3AI* marker was lost between fourth and seventh restreak in the second type II survivor. (C) and (D) Telomere length reverts to wild type in type I and type II survivors after reintroduction of telomerase. Plasmid pRS317-*TLC1* was transformed into both type I and type II survivors. Transformants were restreaked multiple times. Genomic DNA from the first and fourth restreak from a type I survivor and from the first, fourth, seventh, tenth, thirteenth, sixteenth and nineteenth restreak from a type II survivor was digested with *Xho*I (C) or with a combination of *Alu*I, *Hae*III, *Hin*fI and *Msp*I (D), fractionated through a 1% agarose gel, and analyzed by Southern blotting using a $C_{1\text{-}3}A/TG_{1\text{-}3}$ probe. DNA from wild-type and from the two survivors before introduction of the pRS317-*TLC1* plasmid were also analyzed. Similar results were obtained with three additional type II survivors. Size markers are in kb. The $C_{1\text{-}3}A/TG_{1\text{-}3}$ hybridizing fragments in panel C that are >0.5 kb and that are absent in DNA from the type I survivor after telomerase was reintroduced are from X telomeres. Not all type I survivors lacked X telomeres after reintroduction of *TLC1* (data not shown).